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[However], there is no evidence of record to support any of the asserted uses of the instant specification and one of ordinary skill in the art would not find any of the asserted uses of the specification specific, substantial and credible for the reasons of record.

*See*, Paper No. 35, page 3, first paragraph.

Applicants respectfully disagree and traverse.

Applicants assert that a specific and substantial utility is explicitly identified for the claimed invention. As detailed in the response submitted on December 19, 2001, Applicants reiterate that VIGF (also known as ESM-1 or endocan) can be used as a tumor diagnostic. *See, e.g.*, specification at page 27, final paragraph through the top of page 28. As asserted in the specification, "VIGF protein expression may be linked to vascular disease or neovascularization associated with tumor formation." *See*, specification at page 27, final paragraph. Moreover, Bechard et al. (2001) also support the asserted function of VIGF (referred to therein as endocan) as a tumor diagnostic: "[T]he concentration of endocan was found to be elevated in the sera of patients with lung cancer." *See*, J Biol Chem 276:48341-48349 at page 48348, second column, last two sentences of last full paragraph (reference AI in the Supplemental Information Disclosure Statement submitted December 19, 2001). Although, as the Examiner notes, Bechard et al. (1998) also teach that ESM-1/endocan is also found in the sera of healthy patients and increased in patients with acute or severe sepsis (J Immunol 167:3099-3106), this does not render moot the fact that ESM-1/endocan is also useful as a tumor diagnostic in lung cancer patients. Hence, the specification clearly discloses specific, substantial and credible assertions of utility of the disclosed polynucleotides (and encoded polypeptides) as a tumor diagnostic.

Moreover, Aitkenhead et al. (2002) (a copy enclosed herewith) also provide additional support for VIGF (therein referred to as ESM-1) as a useful tumor diagnostic (Microvasc Res 63:159-171). In this report, Aitkenhead et al. demonstrate a correlation between ESM-1 expression and tumor angiogenesis. It is well known in the art that aggressive tumors are often associated with high levels of angiogenesis. "Thus, ESM-1 levels correlate very well with one measure of tumor aggressiveness and vascularity." *See*, Microvasc Res 63:159-171 at pages 165-166, second column, last sentence.

In light of the supporting evidence, Applicants submit that a correlation between the claimed protein and its usefulness as a tumor diagnostic, as originally asserted in the specification, has been established.

In view of the above, Applicants submit that the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101. Accordingly, Applicants respectfully assert that the rejection of the claims 54-67, 75-92, 102-107, 115-119 and 122-175 under 35 U.S.C. § 101 has been obviated and request that the rejection be withdrawn.

### **Rejections Under 35 U.S.C. § 112**

The Examiner has also rejected claims 54-67, 75-92, 102-107, and 115-119, and 122-175 under 35 U.S.C. § 112, first paragraph, as allegedly failing to adequately teach how to use the instant invention for the reasons given with regard to the rejection of these claims under 35 U.S.C. § 101.

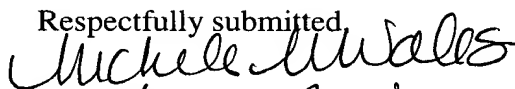
Applicants respectfully disagree and traverse.

For the reasons discussed above in response to the rejection under 35 U.S.C. § 101, Applicants submit that the claimed invention is supported by a specific and substantial or well-established utility. Moreover, this immediate and specific utility is explicitly taught in the specification as filed. The Examiner “should not impose a 35 U.S.C. § 112, first paragraph, rejection grounded on a ‘lack of utility’ basis unless a 35 U.S.C. § 101 rejection is proper.” M.P.E.P. § 2107(IV) at 2100-28 (Rev.1, Feb. 2000). Therefore, since the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejection of claims under 35 U.S.C. § 112, first paragraph, based on lack of utility of the claimed invention, should be withdrawn.

**Conclusion**

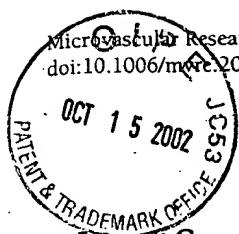
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# Identification of Endothelial Cell Genes Expressed in an *in Vitro* Model of Angiogenesis: Induction of ESM-1, $\beta$ ig-h3, and NrCAM<sup>1</sup>

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Blood vessel growth by angiogenesis plays an essential role in embryonic development, wound healing, and tumor growth. To understand the molecular cues underlying this process we have used the PCR-based subtractive hybridization method, representational difference analysis, to identify genes upregulated in endothelial cells (EC) forming tubes in 3D collagen gels, compared to migrating and proliferating cells in 2D cultures. We identified several previously characterized angiogenic markers, including the  $\alpha_v$  chain of the  $\alpha_v\beta_3$  integrin and plasminogen activator inhibitor-1, suggesting overlap in gene expression between tube-forming cells *in vitro* and *in vivo*. We also found a 2- to 10-fold upregulation of  $\beta$ ig-h3 (a collagen-binding extracellular matrix protein), NrCAM (a "neural" cell adhesion molecule), Annexin II (a tPA receptor), ESM-1 (an EC-specific molecule of unknown function), and Id2 (an inhibitory bHLH transcription factor). We identified a novel splice variant of the ESM-1 gene and also detected dramatically enhanced expression of ESM-1 and  $\beta$ ig-h3 in several tumors. Antisense oligonucleotides to  $\beta$ ig-h3 blocked both gene expression and

tube formation *in vitro*, suggesting that  $\beta$ ig-h3 may play a critical role in EC-matrix interactions. These data expand the suite of genes implicated in vascular remodeling and angiogenesis. © 2002 Elsevier Science (USA)

**Key Words:** angiogenesis; gene expression; RDA; antisense.

## INTRODUCTION

Angiogenesis, the development of new blood vessels by sprouting from the preexisting vasculature, plays an important role in a number of physiological and pathological processes. Significant blood vessel growth has been shown to accompany organ development during embryogenesis, formation of the corpus luteum, placentation, wound healing, and tumor formation (Conway *et al.*, 2001; Yancopoulos *et al.*, 2000). The growth of new blood vessels accompanying tumor development has, in particular, lead to greater interest and improved understanding of the central role of the endothelial cell (EC) during angiogenesis.

After initial activation by angiogenic mediators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), EC degrade the local basement membrane, migrate into the underlying

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ing stroma, proliferate, and form capillary sprouts. It is believed that opposing sprouts coalesce and form a new capillary loop (Folkman, 1985). However, these events are rare in normal, healthy, adult tissues and most EC are quiescent, subject only to short, tightly regulated bursts of angiogenic activity. It has become increasingly clear that these brief periods of EC activation are regulated by a balance of pro- and anti-angiogenic forces (Hanahan and Folkman, 1996; Risau, 1997). In normal adult tissues, anti-angiogenic mediators are dominant and EC are suppressed and quiescent. During angiogenesis, the release of molecules, such as VEGF, overwhelms the inhibitors and EC undergo the morphological steps necessary to generate a new capillary loop.

The molecular cues underlying these morphological changes are, in large part, unknown. Some of these events can be modeled, however, using one of a range of *in vitro* assays. These assays test the ability of putative angiogenic mediators to induce EC to migrate, proliferate, or form tube-like structures. EC embedded in type I collagen undergo rearrangement to form capillary tube-like structures (Madri *et al.*, 1988; Montesano *et al.*, 1983). These structures are morphologically similar to the primary capillary plexus that is formed after the blood island stage of embryonic development. This assay models the EC migration, alignment, and interaction with the extracellular matrix (ECM) that occurs during angiogenesis. The initial stages of angiogenesis are characterized by both matrix degradation and deposition. Collagen deposition seems particularly important: inhibition of collagen assembly and deposition prevents angiogenesis (Haralabopoulos *et al.*, 1994), while collagen type I knockout mice die *in utero* with malformed and ruptured blood vessels (Löhler *et al.*, 1984). The collagen gel model has been used extensively to study the angiogenic/anti-angiogenic potential of molecules such as insulin-like growth factor (Nakao-Hayashi *et al.*, 1992), leukemia inhibitory factor (Pepper *et al.*, 1995), VEGF-D (Marconcini *et al.*, 1999), bFGF-induced plasminogen activator (Giuliani *et al.*, 1999), and connective tissue growth factor (Shimo *et al.*, 1999). In addition, signal transduction pathways have been investigated using specific activators and inhibitors of signaling molecules (Ilan *et al.*, 1998). However, it is

still the case that while the interaction of EC with ECM molecules and the activity of pro- and anti-angiogenic molecules on EC *in vitro* are well documented, considerably less is known about subsequent downstream gene expression and activation.

The aim of the present study was to identify the gene profile expressed by EC forming tubes in a 3D collagen gel. We isolated cDNA from tube-forming EC and from EC migrating and proliferating on top of a collagen gel, both grown in the presence of bFGF and VEGF, and enriched for genes differentially expressed in tubes using the PCR-based subtractive hybridization method, representational difference analysis (RDA). Our assay, therefore, selects for genes expressed in response to the combination of growth factors and extracellular environment rather than in direct response to growth factors alone. Our findings indicate that well-known angiogenic genes are differentially expressed during tube formation *in vitro*, suggesting that the 3D collagen gel assay models at least some aspects of angiogenesis. Furthermore, we have identified several genes that have not been previously implicated in angiogenesis.

## MATERIALS AND METHODS

### Cell Culture

Human capillary endothelial cells (HUCE or EC) were prepared from liposuction adipose tissue using anti-CD31-coated magnetic beads as described previously (Springhorn *et al.*, 1995). Tissue was obtained under protocols approved by the appropriate IRB committees. A 10-ml vial of collagen gel mixture was made by adding 1 ml 10× Hanks' balanced salts (Gibco, Grand Island, NY) to 4 ml 50 mM Hepes (pH 8.5) over ice. After the Hanks'/Hepes mix was supplemented with 30 mg fibronectin (Sigma, St Louis, MO), 5 ml of 3 mg/ml rat tail collagen type I was added to give a final solution of 1.5 mg collagen/ml. This mixture rapidly polymerizes to form the collagen gel when warmed to 37°. Rat tail collagen was prepared according to the method of Schor *et al.* (2001). In our experience, better tubes are formed in the presence

of fibronectin. To induce capillary formation,  $1 \times 10^5$  HUCE were seeded onto a thin layer of collagen gel in the well of a 24-well plate, allowed to adhere for 1 h, and then overlaid with a second layer of collagen (0.5 ml) to provide a 3D matrix. Cells were grown in Medium 199 supplemented with 20% FBS, 20 ng/ml recombinant human VEGF (Genzyme, Cambridge, MA), and 20 ng/ml recombinant human bFGF (R&D Systems, Minneapolis, MN). These 3D gel EC are referred to as tube-forming EC or tubes. Cells were also grown directly on the bottom layer of collagen gel in the same medium (2D culture). Importantly, both the 2D and the 3D cells were cultured in identical medium, containing bFGF and VEGF.

### Representational Difference Analysis

For RDA, EC were harvested after 18 h in culture using 0.4% collagenase I (Worthington, Lakewood, NJ) and collected by centrifugation. RNA was prepared using RNA isolation kits (Stratagene, La Jolla, CA). Poly A<sup>+</sup> mRNA was purified over oligo dT columns and reverse transcribed into double-stranded cDNA using a cDNA synthesis kit from Stratagene. Tester representation (amplified cDNA from tube-forming EC) and driver representation (amplified cDNA from 2D EC) were subjected to three rounds of subtractive hybridization exactly as described (Henderson *et al.*, 2001; Hubank and Schatz, 1999), using ratios of 1:100, 1:800, and 1:400,000.

### Cloning and Characterization of Difference Products

The second and third difference products (DPII and DPIII) were digested with *DpnII* and cloned into the *Bam*HI site of pBluescript II KS<sup>+</sup> (Stratagene). Transformants with inserts were identified by blue/white screening. To identify duplicate clones, colonies were grown on PVDF membranes using 96-well replicas (one for each round of hybridization) and the membranes were probed sequentially with inserts cut from individual clones. Doubled-stranded cDNA cut from representative clones was radiolabeled to probe virtual Northern blots containing tester and driver cDNA populations. This allowed a first estimation of differential expression between tester and driver to be as-

certained. Blots were exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) and band intensities were measured using ImageQuant software (Molecular Dynamics). After normalization to GAPDH, relative increases between tester and driver representations were calculated.

### Northern Blotting and RT-PCR

For Northern blotting, poly A<sup>+</sup> mRNA was prepared from tubes and 2D cultures and resolved on standard formaldehyde gels. After crosslinking to nylon membranes, blots were hybridized to radiolabeled probes using ExpressHyb (Clontech, Palo Alto, CA) at 68° or Ultraspeed (Ambion, Austin, TX) at 42°. Multiple tissue Northern (MTN) blots (Clontech) and a matched tumor/normal tissue array (Clontech) were hybridized with ExpressHyb at 68°. After hybridization, blots were washed and developed according to the manufacturer's instructions. Blots of tubes and 2D cultures were imaged and quantified on the PhosphorImager. MTN blots were exposed to Kodak X-ray film. The tumor array was imaged and quantified on the PhosphorImager. Spot intensities for each of the probes were statistically analyzed with Kendall's rank correlation, using StatView 5.0 (SAS Institute, Inc., San Francisco, CA).

For RT-PCR, 2 µg of total RNA was prepared from tube-forming and 2D EC, random primed, and reverse transcribed with Superscript II (Gibco). Specific primers for ESM-1 (upper 5'gctaccgcagctctcagg3' and lower 5'attgcatttttagtcttgagtgt3') and GAPDH (upper 5'accacagtccatgccatcac3' and lower 5'tccaccctgttgctgtgtgctgta3') were used. After a hot start at 94°, primers were annealed for 45 s (ESM-1 at 55°, GAPDH at 60°) and extended for 45 s at 72°. PCR was performed for 30 or 40 cycles.

### Antisense Assay

Sequence-specific antisense oligonucleotides were designed using the program "Oligo" (National Biosciences, Plymouth, MN). Two of six sequences designed for  $\beta$ ig-h3 are reported here: -696 5'gtggctcgctttcaggag3' and -1263 5'gtcaaccgctcacttcca3'. For the controls the following sequences were used:

VEGFR2 5'cggactcagaaccacatc3' and nonsense control 5'ccctccctgttactccc3'. All the nucleotides were sulfate-substituted to avoid degradation by nucleases (S-oligonucleotides). Oligonucleotides were tested at various doses ranging from 0.03 to 3  $\mu$ M and their ability to block tube formation in collagen gel cultures was assayed in 24-well plates. EC were preloaded with oligonucleotides by osmotic shock using Influx (Molecular Probes). In each well,  $1 \times 10^5$  EC were embedded in collagen as before. Assays were performed in triplicate and cultures were fixed in 4% paraformaldehyde/PBS 18 h after plating. After fixation, each well was scored independently by two highly skilled individuals with no prior knowledge of the arrangement of the assay and the degree of tube formation was graded in five levels from 0 to 4. The scale used was 0, monolayer; 1, some endothelial cell elongation; 2, tube formation with some branching; 3, extensive tube formation and branching; 4, extensive lattice formation. Statistical analysis was performed using a Student *t* test. To demonstrate the specificity of the antisense molecules, total RNA was extracted from the tube-forming EC and reduction in  $\beta$ ig-h3 mRNA levels was confirmed by RT-PCR with specific primers (upper 5'tgacctcctggctccctgaat3' and lower 5'gcccgatgcctcgcctaac3') as before (see above) with an annealing temperature of 60°.

## RESULTS

### Identification of Induced Genes

EC were plated on top of a collagen gel or within a collagen gel sandwich and cultured in medium containing bFGF and VEGF. After 18 h, EC embedded in the 3D collagen formed an extensive network while those plated on top of collagen formed a regular monolayer (Fig. 1). In the gels EC become elongated and fused with their neighbors to form an intricate, capillary-like web. Large open spaces were visible between the cells (Fig. 1). Frozen sections cut perpendicular to the plane of the network revealed lumens in many of the cross-sections (Henderson *et al.*, 2001, and data not shown).

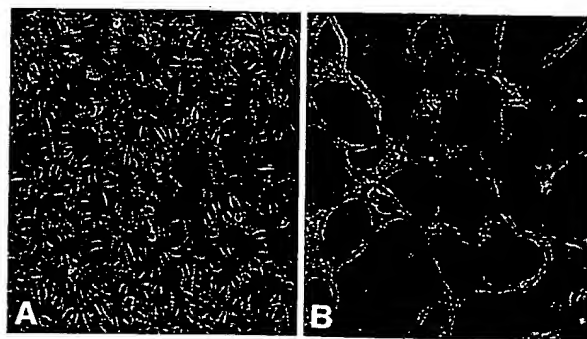


FIG. 1. EC tube formation. (A) EC grown on a layer of collagen (2D) form typical monolayers. (B) EC cultured between two layers of collagen (3D) form elongated tube-like structures. Both cultures contain bFGF and VEGF at 20 ng/ml. Sections cut perpendicular to the plane of culture revealed the presence of lumens (Henderson *et al.*, 2000, and data not shown). Original magnification  $\times 400$ .

After performing three rounds of RDA, DPII and DPIII cDNA fragments were cloned into pBluescript and sequenced. It was anticipated that DPIII clones would only be expressed in the tester representation and that some genes of interest, expressed at low levels in 2D cultures and upregulated in the tube-forming EC, would be thereby excluded. To circumvent this potential problem, we decided to also examine DPII clones. Sequences were queried against GenBank using BLAST (Table 1). The most common gene isolated was spermine/spermidine N1-acetyl transferase. This housekeeping gene is involved in recycling of polyamines and is expressed by cells when they stop proliferating. Another common cDNA enriched during the RDA encoded a novel bHLH transcription factor HESR1, which has been described elsewhere (Henderson *et al.*, 2001). HESR1 is necessary for proper tube formation *in vitro* and modulates EC expression of the VEGF receptor flk-1/KDR (VEGFR2).

Most of the remaining clones identified in the DPII were isolated only once or twice in the 65 clones sequenced. Many of these clones encoded for housekeeping proteins that appear to be upregulated during tube formation. The expression pattern of this class of molecules was not examined further.

In large open-ended screens such as this, it is critical to have secondary and tertiary screens that both confirm the differential expression and reduce the number of genes to be analyzed in greater depth. We have used Northern blots and RT-PCR to confirm differen-



TABLE 1  
Differentially Expressed Genes Identified by RDA

Gene	GenBank Accession No.	No. of hits	Cloned from	Fold increase in tester over driver
Spermine/spermidine N1-acetyl transferase	NM_002970	14	DPII/III	7.0
HESR1	AF151522	12	DPII/III	8.0
Connexin 40	AF151979	4	DPII/III	3.0
ESM-1	NM_007036	4	DPII	4.2
Very long chain acyl CoA dehydrogenase	D43682	2	DPII	ND
KIAA0850 (NS1-BP)	XM_001739	2	DPII	ND
Carbonic anhydrase II	XM_005208	1	DPII	ND
$\alpha_v$ integrin	XM_005208	1	DPII	3.0
Pre-chondroitin sulfate	U16036	1	DPII	3.0
Endothelial plasminogen activator inhibitor	X04429	1	DPII	3.5
3-5 cAMP phosphodiesterase	XM_001862	1	DPII	ND
Annexin II	XM_015855	1	DPII	1.7
NrCAM	XM_004950	1	DPIII	9.8
Cytochrome P450 (CYP1a1)	XM_007727	1	DPII	ND
ADAM-10	XM_007741	1	DPII	ND
Big-h3	M77394	1	DPIII	5.3
Id-2	XM_002273	1	DPII	5.0
General transcription factor Ili	XM_011605	1	DPII	ND
KIAA0911	AB020718	1	DPII	ND
KIAA0538	AB01110	1	DPII	ND
Unknown (A4)		1	DPII	1.8
Unknown (G7)		1	DPII	1.6

tial expression and then used tissue expression studies and, finally, functional assays to focus in on a small subset of genes that are likely to be of greatest interest. These studies are described below.

### Confirmation of Differential Expression by Virtual Northern

We next selected several genes for further analysis. As a preliminary screen to confirm differential expression of these genes, the cDNA fragments were radio-labeled and used to probe a virtual Northern of tester and driver cDNA. Blots were analyzed on a Phosphor-Imager and intensities of the bands normalized to GAPDH (Fig. 2A).

Several genes that have been implicated in angiogenesis were confirmed as being differentially expressed in tubes. Expression of the  $\alpha_v$  integrin was 3-fold higher in the tester (tubes) than the driver cDNA. Similar increases in expression were observed with plasminogen activator inhibitor 1 (PAI-1) and Annexin II (AnnII) (3.5- and 1.7-fold increases, respec-

tively). Levels of the glycoprotein building block, pre-chondroitin sulfate, were also raised 3-fold in tubes.

Some of the most significant increases in expression were observed in a group of known genes that have not previously been shown to have angiogenic function. Interestingly, NrCAM, which is important in neural development, was expressed by EC. Expression of NrCAM in tubes was almost 10 times greater than in the 2D EC. ESM-1, previously identified as being highly expressed in lung EC, was upregulated four-fold, while  $\beta$ ig-h3, a collagen-binding protein found in vessel walls, was overexpressed fivefold in tubes. Id-2, which blocks transcription of genes containing E boxes in their promoters by sequestering bHLH transcription factors, was upregulated fivefold in tubes.

Two novel genes, A4 and G7, were also identified in this assay. Adjacent expressed sequence tags from the dbest database were aligned to form contigs of 815 bp and 2.6 kb for A4 and G7, respectively. The G7 contig is 50% homologous at the amino acid level to protein disulfide isomerase while A4 appears to be a new member of the 5' nucleotidase family (48% homology

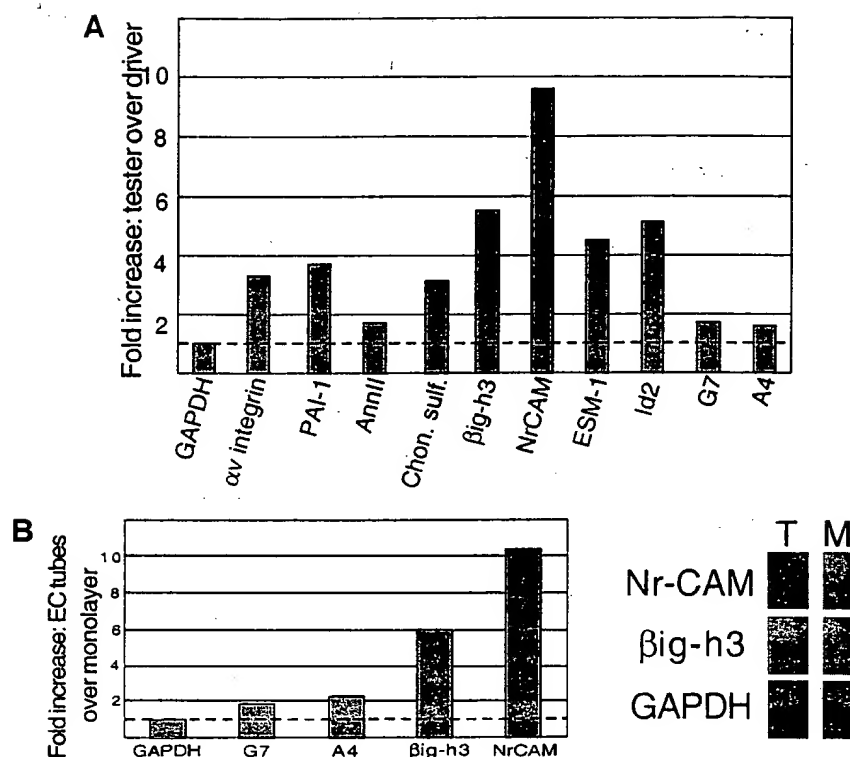


FIG. 2. Differential expression of identified clones in the tester and driver cDNA representations and on Northern blots. (A) Fold increases in expression in the tester over the driver were quantified on a PhosphorImager and normalized to GAPDH. (B) Differential expression of  $\beta$ ig-h3, NrCAM, and the novel genes, A4 and G7, in tube-forming EC versus monolayers, was confirmed by Northern analysis. Representative Northern blots are shown for NrCAM and  $\beta$ ig-h3. The blot was quantified on a PhosphorImager and expression was normalized to GAPDH. In both (A) and (B) the dotted line represents equal expression in monolayers and tubes.

to purine 5' nucleotidase). Differential expression of these novel genes was 1.5 to 2 times greater in the tube cDNA compared to the 2D cultures.

#### Confirmation of Differential Expression by Northern Blot and RT-PCR

When performing subtractive hybridization experiments and gene array studies it is important to confirm differential expression by independent assays. Therefore, we performed Northern blot analysis using mRNA from tube-forming EC and 2D cultures. We probed for  $\beta$ ig-h3, NrCAM, and the novel genes A4 and G7 and normalized the results to GAPDH (Fig. 2B). We found excellent agreement with our virtual Northern data with the largest differences between tubes and 2D cultures detected with NrCAM and  $\beta$ ig-h3 (10 and 5 $\times$ , respectively). Again, only modest

differences were observed with G7 and A4 (Fig. 2B). Northern analysis also allowed a first estimate of the transcript size of the novel genes. Discrete bands were obtained at 3.5 (G7) and 2.0 kb (A4). These genes were not analyzed further.

Differential expression of ESM-1 was confirmed by RT-PCR. At 30 cycles, two discrete bands were detectable in the sample from tube-forming EC, while nothing was visible in EC from 2D cultures (Fig. 3A). By 40 cycles, however, the same two bands were apparent in the 2D cultures (not shown). GAPDH levels were similar from both tubes and monolayers, while no-RT controls demonstrated that no genomic contamination was present. The bands corresponding to ESM-1 were sequenced and indicated the presence of a splice variant, lacking 150 bp (Fig. 3B). Sequencing of the original four ESM-1 clones obtained in the DPII revealed that one of these also had the same splice variant.

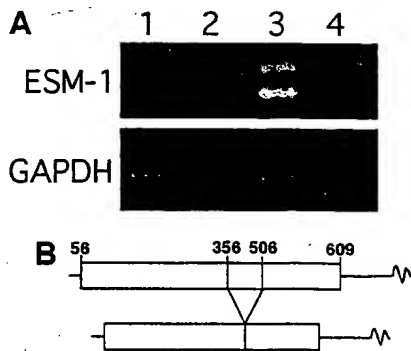


FIG. 3. Differential expression of ESM-1 and identification of a splice variant. (A) Differential expression of ESM-1 in monolayers (lanes 1 and 2) and tube-forming EC (lanes 3 and 4) was confirmed by RT-PCR. Using specific ESM-1 primers at 30 cycles, two discrete bands were observed in the sample from EC tubes (lane 3). Samples from monolayers (lane 1) and no-RT controls (lanes 2, 4) were blank at 30 cycles. Both bands from lane 3 were sequenced; the upper band contains a fragment that corresponds exactly to the published ESM-1 sequence while the lower band has 150 bp deleted. GAPDH levels were similar from monolayers and tubes (lanes 1 and 3). (B) Schematic of ESM-1 including the splice variant identified in the RT-PCR and identified in one of four ESM-1 clones in the RDA. Numbering refers to nucleotides as defined by Lassalle *et al.* (1996).

Interestingly, the open reading frame is maintained in the splice variant, suggesting that a distinct protein form of ESM-1 may be expressed, lacking an internal stretch of 50 amino acids. Future studies will determine the tissue specificity of the splice variant and whether it has a specific role to play in angiogenesis.

#### Tissue Expression of Differentially Expressed Genes

At this point we narrowed our analysis down to  $\beta$ ig-h3, NrCAM, and ESM-1, based on their strongly upregulated expression in tubes. To examine tissue expression of these genes we probed a MTN blot (Fig. 4). After extended exposure (9 days), bands corresponding to ESM-1 were detected in kidney and lung. All other probes were exposed for 24 h. NrCAM was strongly expressed in brain and weakly in placenta, whereas  $\beta$ ig-h3 was detected in all tissues except brain, the highest intensities being in heart, placenta, and kidney.

Gene expression was further examined in matched pairs of normal and tumor tissue. We were unable to

detect NrCAM in any tissues on the blot, suggesting that expression levels may be very low throughout the tissue, or they may be high, but only in very few cells (see discussion). There were no brain tumors represented on the blot, a tissue where NrCAM expression would be expected. ESM-1 was found to be dramatically upregulated in several (5/14) renal cell carcinomas; however, expression appeared to be somewhat higher in normal tissue in other samples (Fig. 5A). We rehybridized the blot with a probe for the housekeeping gene ubiquitin (Ub) and, using PhosphorImager data, calculated Kendall rank correlations between each probe for pooled normal and tumor specimens. As shown in Fig. 5B, no significant correlation was found between the intensity rankings for ESM-1 compared to Ub, indicating that the upregulated ESM-1 expression in tumors is not due to differences in loading of the cDNA target on the blot.

Aggressive tumors are marked by high levels of angiogenesis, which has been used as a prognostic indicator (Tas *et al.*, 2000). Similarly, high expression of VEGF by tumors has been linked to poor clinical outcome (Yuan *et al.*, 2001). In light of this we hybridized the blot with a VEGF probe and again performed correlation analysis. Here we found a very strong correlation between ESM-1 and VEGF, but again, no correlation between VEGF and Ub. Thus, ESM-1 levels

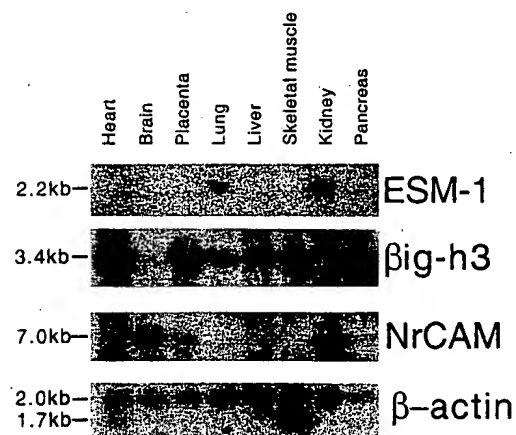


FIG. 4. Tissue distribution for NrCAM,  $\beta$ ig-h3, and ESM-1. A multiple tissue Northern blot was sequentially probed with specific radiolabeled cDNA. All blots were exposed for 24 h except ESM-1, which was exposed for 9 days.  $\beta$ -Actin levels were roughly equivalent in all lanes.

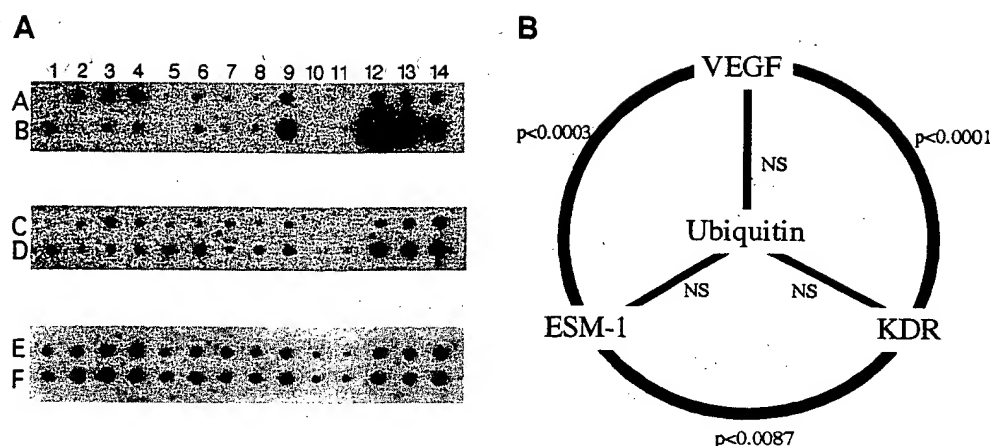


FIG. 5. (A) Expression of ESM-1 in kidney tumors. Dot blot of 14 matched normal kidney (row A) and renal cell carcinoma (row B) cDNA probed for ESM-1. Normal tissue was removed from uninvolved sites adjacent to the tumor. Note the increased expression of ESM-1 in the tumor samples in lanes 1, 9, 12, 13, and 14. The dot blot was reprobed for VEGF (rows C and D), VEGFR2 (data not shown), and ubiquitin (rows E and F). Note the similar expression pattern for ESM-1 and VEGF and the contrast with Ubiquitin. (B) Statistical correlation among ESM-1, VEGF, VEGFR2, and ubiquitin. Kendall's rank correlation determined the degree of similarity in the expression patterns of the genes in the renal cell carcinoma samples. The schematic illustrates the generated *P* values between genes. Note the strong relationship among ESM-1, VEGF, and VEGFR2, but nonsignificant (NS) correlation between these genes and ubiquitin.

correlate very well with one measure of tumor aggressiveness and vascularity. Finally, as a further measure of angiogenesis and vascularity in ESM-1- and VEGF-high tumors, we reprobed for VEGFR2 as a marker of active, angiogenic EC and with CD31 as a pan-endothelial marker. Once again we found a strong correlation between ESM-1 expression and degree of vascularity. We also found very similar correlations between ESM-1 levels and markers of angiogenesis in tumors from breast, uterus, stomach, and rectum (data not shown), indicating that ESM-1 is likely expressed in the vasculature of these tumors also. These data indicate that ESM-1 is upregulated in many tumors and that this may relate to the angiogenic state of the tumor; however, the precise role of ESM-1 in this process and the mechanistic relationship between these proteins is not known.

A similar analysis for  $\beta$ ig-h3 showed a consistent increase in expression in the tumor samples: up in 11/14 kidney, 8/9 breast, 5/7 uterus, 7/11 colon, 8/8 stomach, and 6/6 rectum samples, with increases of up to 70-fold in some cases (data not shown). Again, there was no correlation between Ub expression and expression of  $\beta$ ig-h3. The correlation between  $\beta$ ig-h3

and angiogenic state (VEGFR2 expression) did not quite reach significance, likely reflecting the broader expression of  $\beta$ ig-h3 by stromal cells (O'Brien *et al.*, 1996a), in contrast to ESM-1, which in most tissues is EC-specific (Bechard *et al.*, 2000).

#### Antisense Inhibition of Function

To determine whether NrCAM,  $\beta$ ig-h3, or ESM-1 are necessary for tube formation, we loaded EC with antisense oligonucleotides and assayed the cells for tube-forming ability in collagen gels. Nonsense oligonucleotides were used as negative controls and antisense oligonucleotides to VEGFR2 were used as a positive control. VEGFR2 oligonucleotides at 3  $\mu$ M blocked tube formation by 75% (Fig. 6A). Neither NrCAM nor ESM-1 oligonucleotides blocked tube formation in the collagen gel assay, nor did they affect monolayer cultures (data not shown). This does not rule out a role for these genes in the more complex *in vivo* environment as not all aspects of angiogenesis are modeled in this system—for example, sprouting and anastomosis to other capillaries. Interestingly, however, antisense to  $\beta$ ig-h3 dramatically reduced tube

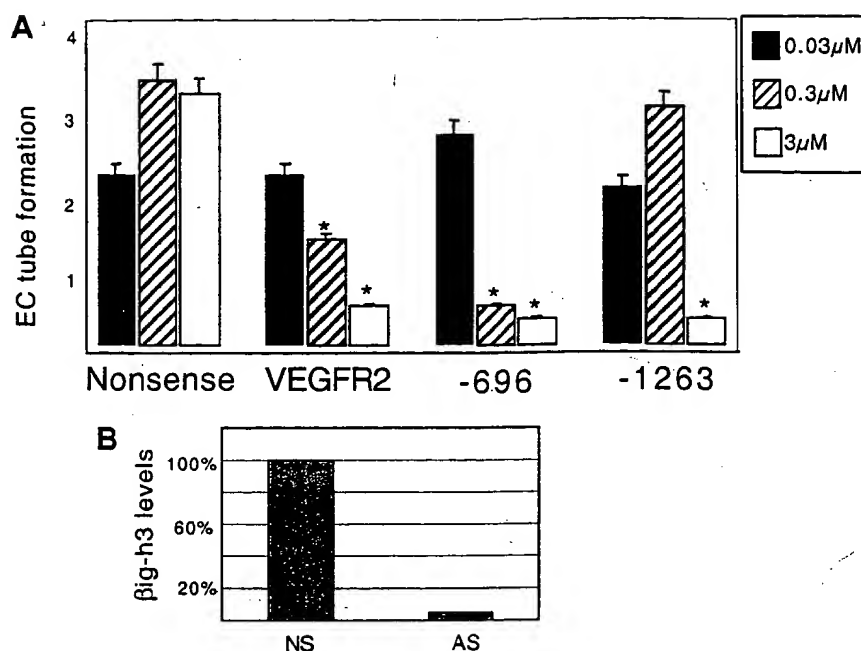


FIG. 6. Antisense blocking of  $\beta$ ig-h3 expression. (A) Targeting  $\beta$ ig-h3 by either antisense molecules AS-696 or AS-1263 resulted in decreased tube formation, compared to nonsense controls. VEGFR2 was used as a positive control. EC were loaded with oligonucleotides at three concentrations (0.03, 0.3, and 3  $\mu$ M) and placed in collagen gels for 18 h. Tube formation was determined by two observers with no prior knowledge of the arrangement of the wells (see Materials and Methods). Each treatment was tested in three C24 wells. All results were similar, both between observers and between experiments. The results shown represent the mean scores of one observer ( $\pm$  standard deviation) in one of two similar experiments. \*Significantly different from control,  $P < 0.01$ . (B) Reduction in expression of target mRNA by antisense was confirmed by RT-PCR (35 cycles) using specific primers. Band intensities were normalized to GAPDH, the expression of which was not altered by either oligonucleotide. Samples from cells incubated with 3  $\mu$ M AS-696 oligonucleotide (AS) had only 5% of the  $\beta$ ig-h3 expression compared to the nonsense control (NS).

formation in collagen gels (Fig. 6A), but had no effect on monolayer cultures (data not shown). The addition of antisense  $\beta$ ig-h3 oligonucleotides resulted in a reduced length of EC tubes and a more poorly developed network compared to nonsense control. For both oligonucleotides shown the effect was dose-dependent, with maximal blocking of over 87% at 3  $\mu$ M for oligonucleotide -696. We confirmed specific reduction of target mRNAs using RT-PCR. In EC treated with the -696 oligonucleotide, we observed a decrease in  $\beta$ ig-h3 transcript of over 95% (Fig. 6B), whereas nonsense oligonucleotides had no effect on  $\beta$ ig-h3 expression. Neither oligonucleotide affected GAPDH expression. Importantly, none of the oligonucleotides used in these experiments contain the GGGG motif, previously shown to be associated with nonspecific effects (Bates *et al.*, 1999).

## DISCUSSION

The collagen gel assay has been used extensively as an *in vitro* model of angiogenesis, where it has been used to study the pro- and anti-angiogenic effects of different mediators. We have used this model to identify genes that are upregulated during the early stages of tube formation as a first step to understanding the molecular basis of angiogenesis. A number of the genes we have identified have been shown previously to be angiogenic mediators, demonstrating the relevance of this model to *in vivo* angiogenesis. Moreover, these data suggest that other genes identified in this assay might also be relevant to angiogenesis. Similar screens have been performed using models that incorporate different extracellular matrix proteins and/or

different combinations of growth factors and PMA (Glienke *et al.*, 2000; Kahn *et al.*, 2000). Alternatively, tumor tissue has been sampled (St Croix *et al.*, 2000). In each case a common set of genes is isolated, which overlaps with our set, along with a suite of genes that are specific to the model being examined. This suggests that seemingly small differences between these model systems can result in subtly different suites of genes being expressed, which is in good agreement with studies showing that angiogenesis in different tissues is not necessarily equivalent (Carmeliet and Jain, 2000).

### Previously Recognized "Angiogenic" Molecules

AnnII and PAI-1, both upregulated in tubes, are members of a large group of molecules implicated in the plasminogen activator (PA)–plasmin pathway (Mignatti and Rifkin, 1996). AnnII provides an EC binding site for tPA and plasminogen interaction, allowing the formation of a complex that permits plasmin formation (Cesarman *et al.*, 1994; Hajjar *et al.*, 1994).

Id proteins are a family of related molecules that, by direct physical interaction, prevent bHLH transcription factors from binding to DNA (Norton *et al.*, 1998). Id2 levels were fourfold higher in tubes than in 2D cultures. A double Id1/Id3 knockout mouse is embryonically lethal with extensive vascular damage, and Id1<sup>-/-</sup>/Id3<sup>-/-</sup> mutant mice fail to vascularize tumor xenografts, clearly suggesting a role for this class of proteins in angiogenesis (Lyden *et al.*, 1999). Interestingly, one of the most frequently isolated sequences from this screen encoded the bHLH transcription factor HESR1, and we have shown that expression of this molecule is necessary for tube formation *in vitro* (Henderson *et al.*, 2001). It is possible that Id and HESR1 may act in concert to permit EC remodeling. The integrin  $\alpha_v$  was upregulated in tube-forming EC, consistent with its enhanced expression during wound healing and tumor formation (Eliceiri and Cheresch, 1999).

### Genes Not Previously Associated with Angiogenesis

Although initially identified as a gene upregulated in lung tumor cells by TGF- $\beta$  treatment,  $\beta$ ig-h3 has

been independently isolated from a collagen-rich fraction of pig cartilage (Hashimoto *et al.*, 1997) and from rabbit cornea (Rawe *et al.*, 1997). This molecule appears to regulate cell to ECM interaction, as chondrocytes and fibroblasts plated onto culture dishes coated with  $\beta$ ig-h3 showed enhanced adhesion and spreading (Ohno *et al.*, 1999).  $\beta$ ig-h3 has an RGD sequence and is likely to bind the  $\alpha_1\beta_1$  integrin, as blocking of other integrins ( $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$  or  $\beta_2$ ) has no effect on cell- $\beta$ ig-h3 interaction (Ohno *et al.*, 1999). Polyclonal  $\beta$ ig-h3 antibodies bind ubiquitously to endothelial cells (O'Brien *et al.*, 1996b), a result that is confirmed in the present study (data not shown). Rawe *et al.* showed by *in situ* hybridization that  $\beta$ ig-h3 message was present in invading EC associated with corneal wound healing but not detectable on normal adult EC (Rawe *et al.*, 1997). This suggests that  $\beta$ ig-h3 is only actively synthesized during vessel remodeling, a proposal supported by immunohistochemical staining of tube-forming EC showing strong staining at the contact zone between the EC and the collagen gel (data not shown). By Northern blot,  $\beta$ ig-h3 mRNA was expressed at very low levels in monolayers but significantly upregulated in tube-forming EC. When the protein and message data are taken together, they suggest that  $\beta$ ig-h3 protein is present on all quiescent EC, probably bound to surface integrins, but is only actively expressed by these cells at low levels. However, once EC *in vivo* become angiogenic or migrate and begin to form tubes *in vitro*, message levels rapidly rise. It is possible that this increased expression of  $\beta$ ig-h3 is necessary to enhance the interaction of EC to ECM during angiogenesis. The reduction in tube formation by cells treated with antisense oligonucleotides to  $\beta$ ig-h3, but not with nonsense oligonucleotides, is entirely consistent with this hypothesis.

Interestingly,  $\beta$ ig-h3 was upregulated in over 80% of the tumors we examined (45/55), although it is likely that expression is not limited to the EC in these tissues. Together with previous publications (O'Brien *et al.*, 1996b; Ohno *et al.*, 1999; Rawe *et al.*, 1997), these data suggest a potentially important role for  $\beta$ ig-h3 in the interaction between angiogenic EC and the ECM.

NrCAM now joins a growing list of "neural" molecules, such as neuropilin-1, ephrin-B2, EphB2, EphB3, and EphB4, that are expressed by EC (Gale and Yan-

copoulos, 1999). Interestingly, NrCAM was undetectable in monolayers but strongly upregulated in tube-forming EC, suggesting a possible role in tube formation and angiogenesis. Wang *et al.* showed NrCAM expression in brain, pancreas, and placenta (Wang *et al.*, 1998), results largely confirmed by MTN in the present study. Recent reports that NrCAM acts as a ligand for axonin-1 suggest a role for NrCAM in neuronal outgrowth and axonal guidance (Lustig *et al.*, 1999). By extrapolation, it is tempting to speculate that NrCAM plays a role in cell-to-cell communication during the directional migration that characterizes the sprouting phase of angiogenesis—a phase not well modeled in the collagen gel assay, potentially explaining the failure of antisense molecules to block in this assay.

Although it was identified in 1996, comparatively little is known about ESM-1. Lassalle and colleagues showed that ESM-1 is an endothelial-specific, secreted protein; modulated by TNF- $\alpha$  and IFN- $\gamma$  (Lassalle *et al.*, 1996). By multiple tissue Northern analysis, ESM-1 is expressed predominately in kidney and lung; however, a more recent report indicates that ESM-1 is also expressed by the vasculature in other tissues (Bechard *et al.*, 2000). ESM-1 has been implicated in immune responses; however, our data suggest but do not confirm that ESM-1 cDNA fragments may also have a role in angiogenesis *in vivo*. Interestingly, three of four ESM-1 in the DPII pool were as published (Lassalle *et al.*, 1996) while one was alternatively spliced. Sequencing showed that the open reading frame was conserved, with identical N and C termini but possessing an internal 50-amino-acid deletion. This is the first report of an ESM-1 splice variant, although the functional significance of this is still to be determined. The deletion does not cover a recognizable motif; however, it has been recently reported that ESM-1 may be a dermatan sulfate proteoglycan and that an o-glycosylation site exists at serine-137 (Bechard *et al.*, 2001), suggesting that our variant may represent a nonmodified form of ESM-1. Future experiments will be required to determine whether this variant is expressed at the protein level. A multiple tumor blot probed for ESM-1 expression revealed a dramatic increase in several tumors, including 5/14 renal cell carcinomas as well as several breast, uterine, and rectal tumors. Al-

though not all tumors appeared to upregulate ESM-1, there was a strong correlation with the angiogenic marker VEGF, as well as with the degree of vascularity. It is possible that ESM-1 is a direct target of VEGF, although we currently have no data to support this suggestion, or it may be an independent marker of tumor angiogenesis in some tissues.

In summary, analysis of genes differentially expressed by tube-forming EC *in vitro* has revealed a suite of well-characterized angiogenic markers, as well as genes whose role in angiogenesis is previously undocumented. The appearance of well-established angiogenic markers such as  $\alpha_v$  integrin and PAI-1 confirms the relevance of this *in vitro* angiogenic assay to *in vivo* systems and lends support to the assignment of other genes identified in the assay to the class of molecules relevant to angiogenesis. In particular, ESM-1, NrCAM, and  $\beta$ ig-h3 are interesting targets for further investigation.

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